

Inflammation and Impaired Adipogenesis in Human White Adipose Tissue

A doctoral thesis by

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**Karolinska
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Cover photo: human subcutaneous white adipose tissue,
which was excised during an abdominoplasty procedure.

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*"There is nothing noble in being superior to your fellow man; true nobility is
being superior to your former self."*

-Ernest Hemingway

*To my late grandmother, Aurelia Pipkins. Without your strength, support,
influence, and inspiration I would be a lesser man.*

Abstract

This thesis aimed to study inflammation and adipogenesis capacity in human subcutaneous white adipose tissue with respect to the development of obesity and associated comorbidities, including insulin resistance.

Study I showed that the transcription factor, MAFB, was associated with increased adiposity and involved in regulation of TNF α -mediated inflammatory response, yet did not seem to directly influence adipogenesis or metabolism in human adipocytes. MAFB expression was upregulated during adipogenesis, and knocking down MAFB mRNA led to reduced TNF α -mediated inflammation. However, MAFB was highly expressed in white adipose tissue (WAT) macrophages, which most likely explains its association with BMI and metabolic syndrome.

Study II identified increased fat cell size as a hallmark of non-obese type 2 diabetic individuals. This hypertrophic status was associated with insulin resistance, inflammation and adipose tissue lipolysis indicating fat cell size to be a marker of pathogenesis. Relative amount of pro-inflammatory macrophages (M1/M2 ratio) correlated positively with fat cell size, lipolysis and TNF α secretion. In addition, early and late adipogenesis markers correlated negatively with fat cell size, suggesting impaired production of new fat cells in WAT hypertrophy.

Study III revealed that human adipocyte progenitors from subcutaneous WAT of healthy individuals consist of a single homogenous cell population. Furthermore, multiple macrophage subtypes were identified. We could not exclude that adipose progenitor subtypes might exist. However, any subtypes were not detectable by the methods employed in this study. Both findings are significant and warrant further investigation.

To conclude the finding in this thesis, we defined a role for MAFB in adipose tissue inflammation, which can possibly serve as a biomarker of insulin resistance and inflammation in WAT. Fat cell size seemed to be the best predictor for insulin resistance in non-obese individuals and this appeared likely connected to impaired adipogenic capacity of WAT. However, a search for different progenitor populations bearing varied abilities to become fat cells through the use of a single-cell RNA sequencing technique was not successful as only one major population of progenitors in healthy WAT was found. This demonstrated a need for further studies in larger cohorts of individuals characterized by different metabolic statuses.

List of Scientific Publications

The following publications form the basis for this thesis. They are the results of three studies (**Studies I-III**) and will be referred to by their roman numbers in Chapters 1 through 6.

I. MAFB as a Novel Regulator of Human Adipose Tissue Inflammation.

Pettersson AM*, **Acosta JR***, Björk C, Krätzel J, Stenson B, Blomqvist L, Viguerie N, Langin D, Laurencikiene J
Diabetologia 2015;58(9):2115–2123

* Authors contributed equally.

II. Increased Fat Cell Size: A Major Phenotype of Subcutaneous White Adipose Tissue in Non-Obese Individuals with Type 2 Diabetes.

Acosta JR, Douagi I, Andersson DP, Bäckdahl J, Rydén M, Arner P, Laurencikiene J
Diabetologia 2016;59(3):560–570

III. Single Cell Transcriptomics Suggest that Human Adipocyte Progenitor Cells Constitute a Homogenous Cell Population.

Acosta JR, Joost S, Karlsson K, Ehrlund A, Li X, Aouadi M, Kasper M, Arner P, Rydén M, Laurencikiene J
Stem Cell Research and Therapy 2017;8(1):250

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List of Abbreviations

ATM	Adipose tissue macrophages
BMI	Body mass index
CCL2	C-C motif chemokine ligand 2
cDNA	Complementary deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
GLUT4	Glucose transporter type 4
hMADS	Human multipotent adipose-derived stem cells
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
MCP1	Monocyte chemoattractant protein 1
MG	Monoacylglycerol
MAFB	Musculoaponeurotic fibrosarcoma oncogene homolog B
mRNA	Messenger ribonucleic acid
NEFA	Non-esterified fatty acids
NFκB	Nuclear factor kappa B
PCR	Polymerase chain reaction
PPARγ	Peroxisome proliferator-activated receptor gamma
RNA	Ribonucleic acid
scWAT	Subcutaneous white adipose tissue
SVF	Stroma vascular fraction
TAG	Triacylglycerol
TNFα	Tumor necrosis factor alpha
WAT	White adipose tissue

Chapter 1

Introduction

1.1 Obesity

Obesity is a preventable and non-communicable disorder defined by excessive body fat accumulation which impairs health. Body mass index (BMI) is a tool used to index body weight in relation to height in order to classify individuals as being underweight, overweight and obese. According to the World Health Organization, normal weight is defined as a BMI < 25 kg/m², overweight as BMI between ≥ 25 and < 30 kg/m² and obesity as BMI ≥ 30 kg/m² [1-4]. In recent history, rates of overweight and obese individuals have grown so rapidly that obesity is considered an epidemic. In 2016, the World Health Organization estimated that 1.9 billion adults in the world were overweight and approximately 650 million adults were obese whereas in 2008 those numbers were 1.5 billion and 500 million, respectively. Obesity is linked to the development of insulin resistance and type 2 diabetes, the prevalence of which has increased considerably [5]. Further, obesity is associated with increased risk for fatty liver disease, hypertension, cardiovascular disease as well as cancer and neurodegenerative disorders like dementia [5-8]. Due to the vast number of obesity-linked pathologies there is considerable need for advancement in the understanding of mechanisms linking obesity and linked complications.

1.2 Overview of white adipose tissue

White adipose tissue (WAT) is a loose connective tissue, which is comprised of many cell types. The predominant population consists of large, lipid storing cells, referred to as adipocytes, which are functionally and morphologically distinct from cells found within the stroma vascular fraction (SVF) of adipose tissue. The SVF contains many cell types including adipocyte progenitor cells (preadipocytes), varied immune cells, fibroblasts and endothelial cells [9-11]. WAT can be found in many regions of the body but in humans it is commonly subdivided into subcutaneous WAT (scWAT) and visceral depots. The

relative importance in metabolism and inflammation between these depots has been a major research focus whereby this thesis primarily focused on scWAT.

Historically, WAT has primarily been implicated in thermal insulation, mechanical protection and energy storage. Thus, under conditions of excess caloric intake, non-esterified fatty acids (NEFA) are taken up and stored in the form of triacylglycerol (TAG). Upon increased energy demand, TAGs are hydrolyzed into glycerol and NEFA, which are then released into the circulation. In target tissues, such as skeletal muscle and liver, NEFA are utilized as an energy source. The view on WAT was profoundly changed in the early 90's when it was demonstrated that it secretes a number of peptides/proteins including leptin, tumor necrosis factor alpha (TNF α) and adiponectin, some of which act via endocrine mechanisms. The term adipokine was coined to describe the factors secreted from WAT in a time-dependent manner [12-15]. Over the years, >600 adipokines have been described in different proteomic screens. The majority of the adipokines exert local effects on the tissue itself via paracrine signaling. Few adipokines exhibit endocrine function and are secreted into circulation where they impact other tissues, e.g. skeletal muscle, pancreas, liver and the central nervous system [16-20]. This draws focus on the potential of WAT to regulate whole-body metabolism as well as explain a role in for WAT expansion effects on other metabolically relevant tissues.

1.3 Functions of the adipocyte

An essential function of the adipocyte is to store TAG through a process termed lipogenesis and release energy in the form of glycerol and NEFA through lipolysis. The balance between these two enzymatic processes is primarily governed by insulin and catecholamines although other hormones may also play a role [21].

1.3.1 Adipocyte insulin response

Islets of Langerhans are home to beta cells, which are the only known cell type to produce, process, and secrete the insulin hormone. In the most simplistic way, elevated blood glucose and incretin levels lead to a depolarization of the beta cells which ultimately lead to increased insulin release. Insulin binds to membrane-bound receptors and elicit signals in many cell types including hepatocytes, myocytes and adipocytes. Consequently, insulin has a prominent role in adipocyte biology where it regulates adipocyte development, metabolism, energy balance, and cell size [22, 23].

Insulin signaling upregulates the production and translocation of specific glucose transporters to the plasma membrane. In adipocytes, this primarily involves the glucose transporter GLUT4. Glucose is converted to dihydroxyacetone phosphate via glycolysis. Thereafter, dihydroxyacetone phosphate is converted to glycerol 3-phosphate by the enzyme glycerol phosphate dehydrogenase [24, 25]. Free glycerol can also be

phosphorylated to glycerol 3-phosphate by glycerol kinase. However, as this enzyme is expressed at extremely low levels in adipocytes, glycerol 3-phosphate is primarily produced via glycolysis. TAGs are subsequently synthesized via lipogenesis where three NEFA are sequentially coupled to one molecule of glycerol 3-phosphate. In order to enable this process, the NEFA need to be activated by the addition of a coenzyme A (CoA) molecule, which is catalyzed by the enzyme fatty acyl-CoA synthetase. Via the glycolytic pathway, glucose can also be used for the synthesis of NEFA in a process termed *de novo* lipogenesis.

1.3.2 Adipocyte lipolysis

Mobilization of fatty acids in adipocytes is carried out by a process termed lipolysis. Stored TAG are hydrolyzed where three NEFA are released from the glycerol backbone in a stepwise reaction. The first step is catalyzed by the enzyme adipose triglyceride lipase (ATGL), which can only hydrolyze TAG to generate one NEFA molecule and a diacylglycerol and requires the co-activator protein CGI-58 for full hydrolase activity [26, 27]. In the second step, the diacylglycerol is hydrolyzed by hormone-sensitive lipase, which generates a second NEFA molecule and a monoacylglycerol (MG) molecule [28-30]. Although hormone-sensitive lipase exhibits both diacylglycerol and TAG hydrolase activity, it displays a highly selective preference to diacylglycerols [31, 32]. In the final step the MG is hydrolyzed by monoacylglycerol lipase [27]. NEFA is released into the blood where they bind albumin for transport to target tissues. Glycerol derived from lipolysis cannot be used locally by the adipocyte itself. Instead it is transported to the liver where it can be phosphorylated by glycerol kinase. Glycerol 3-phosphate can then be used for TAG synthesis, glycolysis or gluconeogenesis in the liver. Furthermore, lipolysis is stimulated by catecholamines and inhibited by insulin. Adrenergic signaling further regulates lipolysis through interaction with catecholamines, where epinephrine and norepinephrine are of most physiological relevance. In adipocytes, Perilipin 1 (PLIN1) coats lipid droplets where TAGs are stored and binds CGI-58 during an unstimulated state which physically occludes enzymatic activity of ATGL. Once catecholamines stimulate lipolysis, protein kinase A phosphorylates PLIN1 inducing a conformational change which thereby releases CGI-58 to activate ATGL [33]. In addition, protein kinase A phosphorylates HSL, which is activated and recruited to lipid droplet surface (27).

1.3.3 Adipocytes in endocrine signaling

Adipocytes and WAT have been shown to secrete a large number of peptide hormones. It is therefore important to consider the possibility of endocrine signaling between adipocytes and other constituent cell types. Adipocytes secrete adiponectin and leptin into the blood, which mediate appetite satiety as well as metabolic and inflammatory functions [20, 34, 35]. Several studies have indicated that adiponectin may potentiate an anti-inflammatory program in obesity and its comorbidities as it has been shown to exert

a positive impact on metabolism, blood pressure, as well as ectopic fat accumulation along internal organs [36-39]. Further, adiponectin has been shown to prevent macrophage foam cell formation [40]. Leptin signals satiety after meals and improves insulin sensitivity in target tissues [34]. It was initially thought to be a potential anti-obesity treatment but subsequent studies showed that leptin is higher in obese subjects, which would be suggestive of a phenotype indicative of leptin resistance [41, 42].

1.4 Adipose tissue morphology and metabolic phenotype

In addition to adipose tissue cytokines, the morphology of the adipose tissue has significant effects on the function of the tissue. With increasing adiposity, it becomes necessary to reorganize the tissue structure to enable adipose expansion. Adipose tissue can expand by either an increase in adipocyte volume or by an increase in adipocyte number.

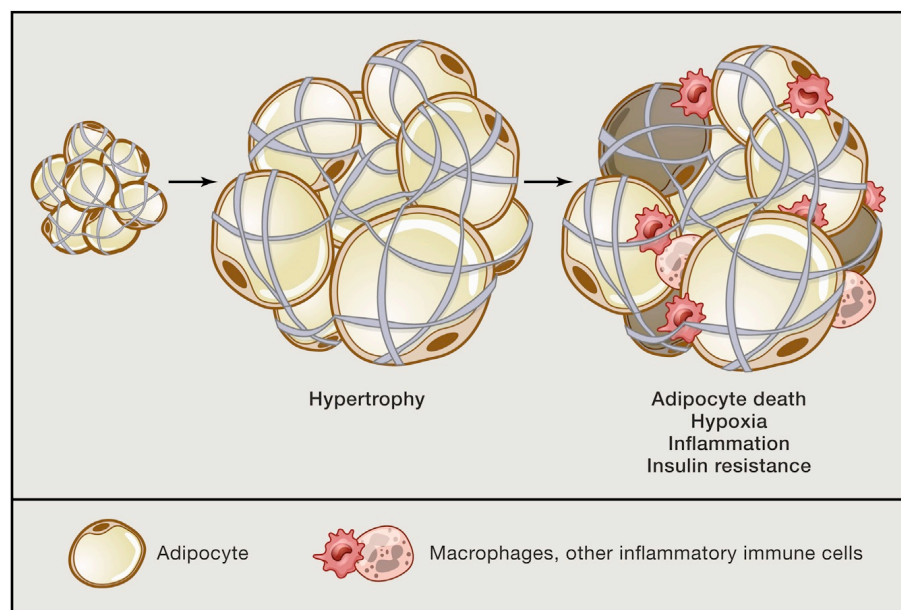


Figure 1.1 Adipose tissue hypertrophy leads to increased inflammation and insulin resistance [43]. Image used with permission from the publisher.

Adipocyte size has been found to correlate with serum insulin and BMI [44-46]. Adipocyte hypertrophy—increased adipocyte size—has been correlated with plasma insulin concentrations and insulin resistance [21, 47, 48]. Moreover, a hypertrophic adipose tissue (Figure 1.1), characterized by fewer but larger adipocytes, is associated with a less favorable metabolic profile consisting of hyperinsulinemia and elevated inflammation in comparison to hyperplastic adipose tissue, which is characterized by more yet smaller fat cells [49]. Morphology differences seems to be of critical relevance in the regulation and function of healthy adipose tissue.

1.5 Stroma vascular fraction

While adipocytes comprise a majority of the WAT mass, they only constitute 20-40% of the total number of cells in the tissue [35, 43]. The SVF is a component of adipose tissue which encompasses the variety of non-adipocyte cells found within the stroma, or structural component of the tissue. The vast majority of these cells are adipocyte progenitors followed by immune cells, which include macrophages, CD8+ and CD4+ T-cells, dendritic cells, B cells, neutrophils and mast cells [9, 50-52]. Studies have suggested that obesity is characterized by an increased number of immune cells in the SVF (primarily macrophages) resulting in a pronounced local enrichment of pro-inflammatory cytokines [52]. The resulting chronic low-grade inflammation promotes the development of insulin resistance and contributes to type 2 diabetes [35, 53-58].

1.5.1 Adipose progenitor cells

Without adipocyte progenitor cells, there would be no adipocytes. These are human adult stem cells, which are multipotent and thereby retain the ability to differentiate into a number of lineage fates including osteocytes, myocytes and adipocytes [59]. With the adipocyte turnover rate at approximately 10% per year [60, 61], WAT mass expansion can be mediated via increased adipocyte number (hyperplasia) and/or size (hypertrophy) [62]. As hypertrophy is closely linked to metabolic complications while hyperplasia is protective, much focus has been given to understanding how adipogenesis is regulated [63, 64]. Much progress has been made in cell and animal models and the major transcription factors and co-regulators have been identified. However, we still lack fundamental knowledge about the mechanisms *in vivo* in man and how these can be modified in a clinical setting.

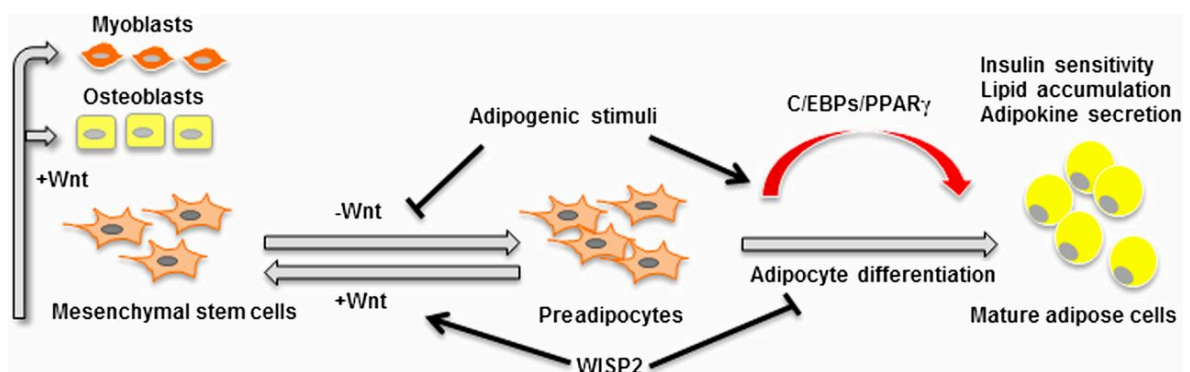


Figure 1.2 Wnt-signaling regulation of adipogenesis. Adapted from Gustafson et. al [67].

Dysregulated adipocyte size has become a focal point of clinical investigation where at present much knowledge is lacking. Adipose hypertrophy is associated with increased risk for type 2 diabetes independent of BMI where non-obese individuals with heredity for diabetes feature increased adipocyte size and insulin resistance, also reported in

Study II [21, 65, 66]. Factors affecting adipocyte size are poorly understood but are often believed to impair adipogenesis whereby Wnt-signaling has been implicated as a regulator of adipocyte differentiation (Figure 1.2) [49, 67-70]. Deciphering what underlying genetic mechanisms perturb adipogenesis and adipocyte metabolism has become a hot topic in adipose research where it is proposed that adipose progenitors are a heterogeneous population [71, 72] which formed the basis for **Study III**.

1.5.2 Adipose tissue macrophages

Adipose tissue macrophages (ATM) are important regulators of adipose tissue inflammation. In the lean adipose tissue ATM are likely to be involved in normal adipose tissue function and turnover by clearing the tissue from apoptotic cells, excess lipid and cellular debris [73, 74]. This population of ATM is referred to as M2-, or alternatively-activated, macrophages, and primarily secretes anti-inflammatory cytokines, e.g. IL10 [75]. In the obese state, it has been thought that monocytes are recruited from the circulation and accumulate in the expanding adipose tissue where they undergo polarization into a class of macrophages with pro-inflammatory phenotype known as M1-macrophages, or classically activated macrophages [75]. M1 ATM release high levels of pro-inflammatory cytokines including TNF α , IL6 and monocyte chemoattractant protein 1 (MCP1) which attract more monocytes from the circulation [57, 58, 76]. Additionally, M1-macrophages also stimulate lipolysis and inhibit insulin signaling in the adipocytes. It is not known what induces the polarization of macrophages *in vivo* but the processes probably involve release of several factors from the adipose tissue itself including exposure to palmitate, an inflammatory fatty acid [54, 77]. Whether adult macrophages are terminally differentiated is a matter of debate. Some studies showed that migration of bone marrow-derived monocytes into tissue was solely related to MCP1 and its receptor, CCR2 [53, 78, 79]. Other studies show that tissue resident macrophages harbor proliferative capacity [80-82] and are derived from the embryonic yolk sac [83-85]. Nevertheless, ATM number increases in obesity and contributes to the inflammation of the adipose tissue [52, 76].

It is important to understand the M1-M2 classification system of macrophages is more often considered to be an oversimplification of a very complex polarization spectrum in which the extremes, M1- and M2- macrophages, are studied. This classification system was the product of *in vitro* stimulated monocytes whereas macrophage phenotypes are variable among various tissues. In adipose tissue, macrophage subtypes have been described with mixed M1-M2 gene profiles [54]. These studies were primarily based on phenotype or cell surface markers that associate with the respective pro-inflammatory or anti-inflammatory phenotypes [86]. Further, the use of specific M1- and M2- macrophage surface markers is not completely consistent between studies. Recently, it has been suggested that there is another pro-inflammatory macrophage phenotype observed only in obese adipose tissue which undergo metabolic activation [77]. Kratz et al suggested that

macrophages might be polarized to a pro-inflammatory state due to a constant presence of high extracellular levels of lipid, glucose and insulin where markers of classical macrophage activation are absent and markers of fatty-acid and cholesterol metabolism are present [77, 87, 88].

1.6 Adipose tissue inflammation

As mentioned above, obesity is associated with a chronic, low-grade inflammation of the adipose tissue and a dysregulation of the tissue function [35, 89]. However, inflammation is not just detrimental as it has been shown to also be advantageous for adipose tissue expansion and remodeling [90]. It is generally considered that altered function of the adipose tissue leads to changes in the storage of NEFA promoting lipid deposition in peripheral organs (lipotoxicity) which in turn increase the risk of developing insulin resistance and non-alcoholic fatty liver disease [89, 91, 92]. Several pro-inflammatory cytokines, such as TNF α , IL6 as well as the MCP1 have been shown to affect WAT phenotype [76]. These factors affect gene transcription via transcription factors and kinases such as NF κ B, MAPK etc. [93]. Several additional transcription factors regulating inflammatory responses have been described in recent years. Among these, V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) is expressed in many tissues in both mice and humans [94, 95].

1.6.1 Tumor necrosis factor alpha

TNF α is an extensively investigated pleiotropic cytokine that affects cellular and biological processes such as immune function, apoptosis, proliferation, differentiation and metabolism [96-98]. The protein is synthesized as a 26 kDa transmembrane protein and reduces to 17 kDa soluble molecule after proteolytic cleavage. The processed TNF α monomers form trimers that bind to ubiquitously expressed transmembrane TNF α receptor 1 and 2. TNF α is a potent cytokine and a strong stimulator of inflammation pathways, (Figure 1.3) [96]. These signals are primarily attributed to an increase in NF κ B activation and increased transcription of pro-inflammatory genes. The role of TNF α with respect to obesity and diabetes was first recognized and defined by Hotamisligil and Spiegelman in 1993 where they showed that adipose tissue mRNA expression of TNF α is induced in rodent models of obesity and diabetes [13]. Increased expression and secretion of TNF α from adipose tissue in obesity had thereafter been confirmed in human [99]. Knockout of the gene encoding TNF α has been shown to restore a degree of insulin sensitivity [100]. Furthermore, the primary source of TNF α within adipose tissue derives from cells in the SVF, namely macrophages [101].

TNF α has been shown to affect adipocyte function and metabolism where it reduces the expression of genes involved in insulin signaling (insulin receptor (IR), GLUT4, and IRS1) or reduction in the adipogenesis master regulator, PPAR γ [12]. PPAR γ is central in

the regulation of genes with critical importance in adipocyte development and function. In addition, TNF α has been shown to activate kinases that promotes phosphorylation of serine residues on the IR and IRS1 which in turn diminishes insulin-induced tyrosine phosphorylation of IR and IRS1 and ultimately suppresses insulin signaling [12].

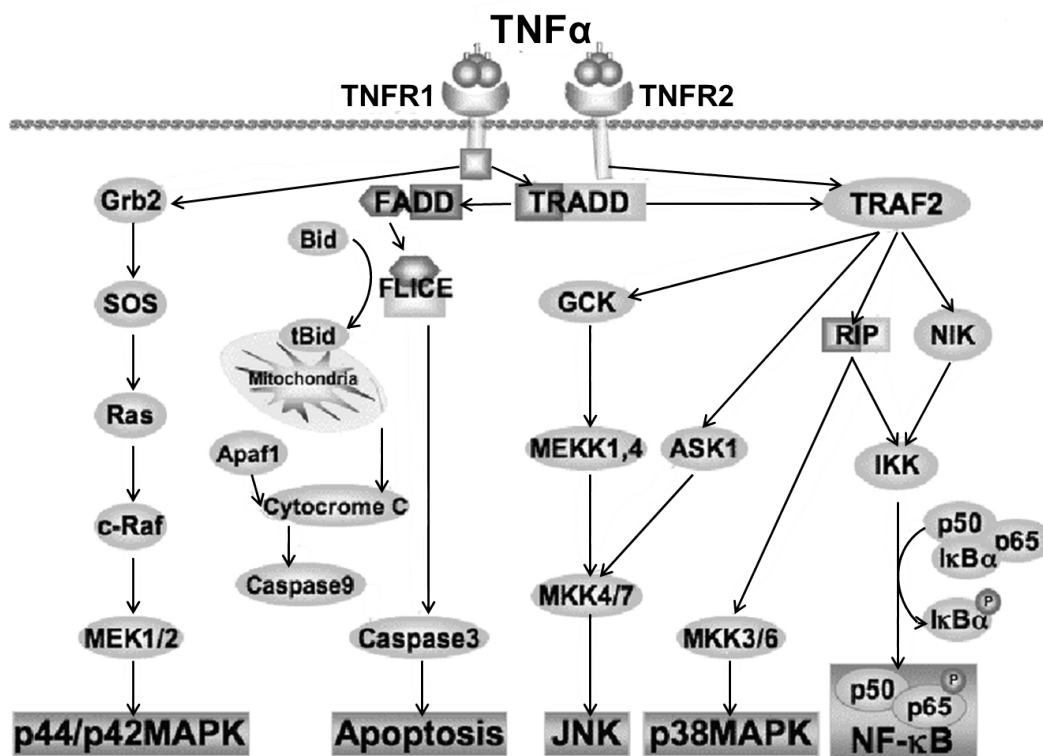


Figure 1.3 TNF α signaling pathway. Inhibitors used in Study I target p44/42 MAPK, JNK, and NF κ B signaling.

1.6.2 Monocyte chemoattractant protein 1

Both mouse models with diet-induced or genetically-induced obesity (ob/ob) as well as obese humans display increased adipose mRNA expression levels of MCP1 (encoded by the *CCL2*) [78, 102-105]. As the name implies, MCP1 is a potent signaling molecule, which promotes migration of monocytes. High levels of MCP1 secretion have been linked to the enhanced infiltration of macrophages into the adipose tissue seen in obesity [104, 105]. Disruption of the *CCL2* gene in mice placed on a high-fat diet leads to a reduced macrophage accumulation, insulin resistance and hepatic steatosis [78]. Moreover, stimulation of murine fat cells with MCP1 reduces insulin-stimulated glucose uptake into the cells [102]. These findings reveal MCP1 as an important link between obesity and obesity-associated metabolic disturbances.

MCP1 is secreted by cells in the stroma vascular fraction, e.g. ATM of adipose tissue as well as by adipocytes, although secretion from isolated adipocytes is significantly lower [78, 105]. Whether MCP1 from adipose tissue is released to the circulation has been a matter of debate and at least in humans, MCP1 primarily works as a local factor

[105]. Nevertheless, MCP1 seems to be an important factor for recruitment of monocytes from the circulation and therefore for the regulation of adipose inflammation.

1.6.3 Musculoaponeurotic fibrosarcoma oncogene homolog B

The transcription factor V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) is a basic leucine zipper transcriptional activator which belongs to the Maf family of transcriptional regulators. MAFB is expressed in many tissues in both mice and humans [94, 95]. MAFB has been shown to regulate fates of many cell types including macrophages, blood cells, beta cells and osteoclasts [106-111]. Through its effects on macrophage differentiation, MAFB may have a role in inflammation. In a human monocyte cell line (THP1), it was demonstrated that TNF α stimulation not only induced MAFB expression but also promoted production of MCP1 [111]. MAFB has been implicated in insulin and glucagon control via transcriptional regulation has been previously implicated in metabolic processes such as insulin and glucagon transcription [112-116]. It was shown that CCAAT/enhancer binding protein δ (CEBP δ) controls transcription of MAFB in keratinocytes, which suggests that MAFB can be regulated by this transcription factor even in adipocytes [106]. Previously, the role of MAFB in the regulation of other metabolic processes in adipose tissue had not been well characterized.

Chapter 2

Aims and Hypotheses

2.1 General aim

Finding new therapeutic targets in adipose tissue which impact obesity, insulin resistance and associated comorbidities is quintessential in the quest to slow the progression of these diseases. This thesis sought to broaden the current understanding of adipocyte and adipose tissue biology as well as adipose inflammation through the use of standardized clinical measures, molecular biology tools as well as the implementation of cutting-edge techniques.

2.2 Specific aims

- I. MAFB is a highly expressed transcription factor that had yet to be characterized in WAT. The aim of **Study I** was to characterize MAFB in adipose tissue with respect to its impact on inflammation, obesity, insulin resistance and adipocyte biology.
- II. Obesity *per se* has a major impact on WAT phenotype. To elucidate the link between adipocyte size and inflammation in type 2 diabetes, and avoid the confounding effects of fat mass, **Study II** investigated WAT cell populations in non-obese men with type 2 diabetes and healthy age- and weight-matched controls.
- III. **Study II** found that increased adipocyte size is a major phenotype of WAT in non-obese T2D. This suggests that disturbed adipogenesis in precursor cells is an important mechanisms linking WAT to type 2 diabetes. **Study III** aimed to map adipose progenitor populations in human WAT by single cell RNA sequencing of SVF.

Chapter 3

Comments on Methodology

3.1 Cell cultures (*In vitro* and *ex vivo* cell systems)

3.1.1 Tissue digestion & stromal vascular fraction isolation

The separation of adipose tissue cell types, adipocytes and SVF, by enzymatic digestion was first described in 1964 by Rodbell [117]. This method utilizes collagenase which breaks the peptide bonds in collagen, a key component of the extracellular matrix. Collagenase II separation of adipocytes and SVF is preferential as it maintains the integrity of insulin receptors [118]. Once total cells are liberated from the tissue, adipocytes separate from the SVF by flotation due density differences owing to their high lipid content. In **Study I**, SVF was subjected to *in vitro* culture directly upon isolation or cryopreserved in FCS supplemented with 10% DMSO. SVF was solely cryopreserved in **Studies II and III** until the time of experimentation.

3.1.2 Cell cultures

Primary Progenitor Adipocytes

Study I utilized primary adipocyte progenitors for *in vitro* experiments. Total SVF was directly cultured in adipogenic media. Adipogenic cells have adhered to the culture dish within 4-6 hours, and, during the course of *in vitro* stimulation, adherent cells without adipogenic capacity, including immune cells, have perished and have been washed away with media changes. The remaining cells are progenitor adipocytes which have begun the adipogenic differentiation program. PPAR γ improves insulin sensitivity and is the principle driver of adipogenesis. In order to stimulate the adipogenic program, Rosiglitazone, a synthetic ligand of PPAR γ , and insulin were included into the adipogenic culture media. Working with primary adipocytes is advantageous as it would reveal most closely what the *in vivo* response to given treatments would be. However, due to the fragility of mature adipocytes *in vitro* they are not suitable for all measures. Therefore,

SVF derived adipocytes have become a highly utilized and informative alternative which retains a degree of biological variability as compared to cell lines.

Human Multipotent Adipose-Derived Stem Cells

Study I utilized a cell line-like primary cell model system for studying adipose progenitors called human multipotent adipose-derived stem cells (hMADS). These cells were developed from primary SVF derived progenitor adipocytes, and expanded *in vitro*. These cells can be differentiated to fully functional adipocytes with respect to lipolytic response, insulin response and cytokine secretion as well as other lineages including myocytes, osteocytes and chondrocytes [119-122]. For expansion, FCS is added to the culture to induce proliferation while the addition of FGF2 maintains adipocyte differentiation capacity. The adipogenic program is stimulated as with primary progenitors by inclusion of Rosiglitazone and insulin into the culture medium. Cultures of hMADSs are relatively easy to work with in comparison to primary adipocyte progenitors and primary mature adipocytes. They are easily cryopreserved and expanded upon thawing. These cells serve well with very complex experimental designs where millions upon millions of cells may be necessary, and when a reduced degree of biological variation is desired.

3.1.3 Human monocytic cell line

The THP1 cell line was used in **Study I**. This immortalized monocyte cell line is derived from the peripheral blood of a 1-year old human male with acute monocytic leukemia. These cells exhibit a large and round morphology with a low degree of aggregation in suspension. Through stimulation with phorbol 12-myristate 13-acetate, these cells can be differentiated from monocytes into M0, or non-activated macrophages [123, 124]. These cells are highly plastic and can yield M1 or M2 macrophage subtypes with appropriate stimuli [124]. These subtypes were not assessed in this thesis as it was beyond the scope of each study; THP1 cells were investigated solely in an undifferentiated state. THP1 cells offer the advantages of ease of expansion and low biological variability. THP1 monocytes are ideal for studies which aim to identify intracellular signals of a particular cytokine or factor, such as **Study I**.

3.2 Lipolysis

In **Study I**, we studied the effects of MAFB knockdown on adipocyte lipolysis. Adipocytes were incubated in culture with and without lipolytic agents for 3 hours. In our studies, the drug isoprenaline was utilized to induce lipolytic response. Isoprenaline is structurally similar to adrenaline, but it acts as an agonist for only β adrenergic receptors. Lipolysis was measured as free glycerol release into conditioned media by a fluorescence reader. This is a preferential to quantifying NEFA molecules as a measure of lipolytic rate

as adipocytes cannot recycle glycerol as they lack glycerol kinase and cannot convert free glycerol to glycerol 3-phosphate, which is essential for NEFA esterification to form TAG [125].

3.3 Gene expression

3.3.1 Quantitative real-time polymerase chain reaction

Conventional polymerase chain reaction took a vast leap forward when thermal cyclers began to incorporate fluorescence detection. Polymerase chain reaction (PCR) transformed from simply amplifying a transcript of a target gene or sequence where the critical output was the quantity of the generated amplicon into a much finer tool. Fluorescence labeling and detection enabled the ability to measure DNA amplification during the log linear phase of PCR amplification and made it a quantifiable measure in real-time, known as real-time quantitative reverse transcription PCR.

qRT-PCR is used to quantify specific transcripts of interest. Briefly, total mRNA is extracted and purified from cells or tissue. It is then reverse transcribed to complementary DNA (cDNA) using random hexamer or oligo dT primers and reverse transcriptase. The cDNA is loaded into the qRT-PCR reaction where it undergoes thermal cycling and transcriptome amplification. Critical components of this measure include Taq DNA polymerase for amplification, a fluorescence label for transcript quantification, and a thermal cycler with fluorescence detection. Gene expression is calculated in relation to stably expressed reference genes, also known as housekeeper genes, which do not vary in the conditions tested, and quantified by the delta-delta Ct method [126].

Studies I and II utilized two widely used chemistries for the detection of transcripts of interest, SYBR Green and Taqman probes.

SYBR Green is a double stranded DNA (dsDNA) binding dye that intercalates nonspecifically into dsDNA polymers irrespective of sequence. When bound to dsDNA, the dye emits light. The essential requirement is that the polymer is dsDNA. Due the nondiscriminatory nature of this dye it detects specific products as well as nonspecific products including primer dimers; this can lead to false positives. This drawback can be overcome with well-designed primers which take into account sequence specificity as well as slight modifications to thermal cycling temperatures to limit unspecific binding. When oligonucleotide primers are designed with care and properly tested by use of melt curves and efficiency testing with cDNA standards, this method can yield highly specific and quantifiable data on gene expression. The cDNA input is relatively low, and the assay is very cost effective.

Taqman® assays utilize a very different fluorescence chemistry altogether. These assays are supplied with a specific probe for the target sequence as well as forward and reverse primers for the sequence of interest. The probe is a oligonucleotide which binds a

specific sequence between the primers. Further, the probe incorporates a reporter dye at the 5' end and a quenching dye at the 3' end. The quenching dye stifles the ability of the reporter dye to emit light when in close proximity to each other. Once the dsDNA is denatured, the temperature is lowered to enable probe and primers to anneal to their specific target sequences. During the next step of the PCR, the Taq DNA polymerase synthesizes new DNA strands from the unlabeled primers and the template cDNA. Once the polymerase reaches the probe, its 5' nuclease activity cleaves the probe, which separates the reporter and quencher dyes enabling fluorescent light emission. Through repeated cycles, the emitted light is proportional to the amount of synthesized target sequence which in turn reflects the amount of starting material. TaqMan® assays are considered the gold standard for quantitative genomic analyses and are well-cited. However, these assays while specific are costly and require larger material input than SYBR Green.

3.3.2 Microarray profiling

Gene expression profiling across the entire genome by microarray provides the possibility to measure and compare the mRNA expression of a very large number of transcripts (>540 000) between different conditions or between specific cell types, as performed in **Study III**. Genome wide mRNA expression measurement and analysis had been a well-established method in comparison with contemporary global mRNA transcript detection technologies, inclusive of RNA sequencing and Cap Analysis of Gene Expression. Clear benefits to utilizing microarray technologies are that they are much more cost effective and input of biological material is minimal. Additionally, there is an option to amplify the mRNA in order to further minimize biological sample input while maintaining robust data quality. Data sets produced are readily comparable between batches however detection limits are present given that the probe set included in the microarray may not bind all available transcripts or binding affinity can be slightly different for specific transcripts, which affects measures of expression level. Thereby, this is a limiting factor of the technology. Data analysis is easily computed with free software packages available in the computing software, R. Further limiting, this technology cannot measure absolute levels of mRNA expression due to varying binding affinities between gene transcripts and respective probes included in the array. Therefore, common practice is to validate key findings with this technology by real-time quantitative reverse transcription polymerase chain reaction, qRT-PCR.

3.3.3 C1 single-cell auto prep integrated fluidic circuit

Study III utilized the C1 single-cell auto prep system for RNA sequencing of individual cells in human SVF. This system operates by way of a microfluidics chip with 96 capture sites designed to retain individual cells with diameters between 10-17 micrometers. SVF cells were prepared in a single-cell suspension and loaded onto the chip. Doublet

discrimination was performed by microscopy and checked by an automated Matlab image analysis algorithm. Lysis, reverse transcription and PCR amplifications were performed using the C1 instrument script. The cDNA quality was assessed by an Agilent BioAnalyzer. Thereafter, cDNA was fragmented and barcoded. Sequencing was performed on an Illumina HiSeq 2000.

Benefits to using the C1 system include ease of operation and an automated workflow. However, there is an inherent bias in that one would need to select an IFC with a particular capture site designed for a specific range of cell diameters, which is a limiting factor. A similar study could have been performed with a less established protocol by way of single-cell flow cytometry based sorting. While the flow cytometry method is a less-well established method for single cell sorting, it offers the potential of high-throughput indexing the cells that are sorted by tagging surface proteins. The C1 system has this capability if coupled with a fluorescence microscope but at a greatly reduced throughput which would in turn affect cell viability prior to cell lysis. This would enable mRNA to be correlated to membrane protein expression. This, however, would introduce bias as a critical limitation at the time was a theoretical maximum of 18 fluorescence channels for sorting. Both options, however, would require the assistance of a bioinformatician, or extensive bioinformatics training, to evaluate the data output.

3.4 Studying protein expression

As mRNA levels do not always reflect corresponding protein expression levels, it is important to measure both when feasible. Protein may not always reflect mRNA levels as mRNA can be degraded before translations, protein can have a short half-life, as well as tight regulation of protein secretion [127, 128].

3.4.1 Western blot

Western blot is a widely used technique for the detection of protein expression and was implemented in **Study I**. Proteins are loaded into wells of a polyacrylamide gel alongside a known protein molecular weight standard. Current is passed through the buffering solution containing sodium dodecyl sulfate (SDS) which causes proteins to undergo electrophoretic shift and maintain a denatured state. Factors which may affect the separation include isoelectric point, molecular weight, and the charge of the protein. When proteins are denatured they shift through the gel and separate by molecular weight where smaller proteins migrate more quickly than larger proteins. Proteins are then electrophoretically transferred to a positively charged membrane. The membrane undergoes blocking and probing with a primary antibody specific to the protein of interest. This is followed by incubation with a secondary antibody specific to the primary antibody to amplify the detection signal. The secondary antibody may be enzymatically labeled or fluorescently labeled whereby detection can be determined by

chemiluminescence or fluorescence detection, respectively. Western blot analysis yields visual evidence of protein expression. Though protein band density may be quantified, it is not a robust measure which can be readily compared between membranes. Additional considerations are that proteins often undergo post-translational modifications which affected the size and charge of the protein, and ultimately, the electrophoretic shift. This can reveal a bands at sizes other than the theoretical or reported molecular weight. Furthermore, proteins may not denature completely and may even degrade which would block or completely destroy the epitope where the antibody would bind. While informative, the outcome of this method is highly variable.

3.4.2 Enzyme-linked immunosorbent assay

Studies I and II utilized Enzyme-linked immunosorbent assays (ELISA) for protein detection. ELISAs are reliable alternatives to the western blot for protein detection. ELISAs are highly quantifiable and can be performed in a fraction of the time it takes to perform a traditional western blot. Protein standard and lysates are loaded onto a microplate pre-coated with antibodies specific to the target protein. Thereafter, the protein is probed with a detection antibody and a secondary antibody which is specific to the detection antibody. The secondary antibody contains an enzyme, often horseradish peroxidase, which will catalyze the substrate solution and change its color. The color change can then be measure with a spectrophotometer, and concentration determined by comparison to the protein standard. ELISA requires much less material than a western blot. Furthermore, conditioned medium from cell or tissue culture can be used to determine levels of protein secretion. A concern is antibody specificity, affinity and cross-reactivity. If the detection antibody is promiscuous then quantification is unreliable. Thus, whenever possible ELISA and Western blot should be used in complement.

3.5 Fluorescence-activated cell sorting

Flow cytometry is a laser based technology which enables the detection of physical and chemical properties of cells which pass through a finely focused fluidic stream at a very high rate. In its purest form, cytometry is used for studying cell characteristics including counting cells, cell size, protein expression, and much more. In principle, as the cells pass through the laser line, they refract light and these light scatter properties are detected. Cells of a specific cell type have very similar light scatter properties, or signatures. In fluorescence based flow cytometry, cells are marked with antibodies with high affinity for specific proteins. The light scatter signatures of each marker incorporated in the staining cocktail and degree of expression by cells in the sample are used for analysis to identify cells with marker expression similarities to define specific cell populations.

This technique enables researchers to obtain highly specific information from many individual cells. **Study III** utilized fluorescence-activated cell sorting (FACS) to

characterize many cell types within adipose tissue and sort specific populations of cells for further investigation. Comparable techniques include fluorescence microscopy and time of flight mass spectrometry (CyTOF). CyTOF was not pursued in this study as it requires access to bioinformaticians for data quality assessment and down-stream analyses. Fluorescence microscopy offers such benefits as being a directly visual assessment, inexpensive by comparison, ease of sample preparation and simplicity of analysis. It falls short of sensitivity when compared to flow cytometry with respect to the number of detection parameters. Flow cytometry has the advantage of yielding large amounts of data which can be relatively easily assessed in a short amount of time. Limitations include spectral overlap between fluorochromes, cellular autofluorescence and the number of parameters which can be evaluated in a given panel. It is of critical importance for the reliability of this technique that specific biological and technical controls be incorporated into the design. These considerations were made during the design of **Study II and Study III**.

3.5.1 Marker selection

Marker selection is a critical step in the design of any flow cytometry based experiment. Ideally, the cell population of interest will express a specific cell surface protein which can be used as a marker of that population while other cell types in the same sample will not express this marker. This is easily adjusted for when performing flow cytometry experiments on *in vitro* stimulated cell lines as these typically encompass one individual cell type where the marker variation is negligible and the output of interest is variation of few parameters. This becomes much more complicated when using heterogeneous samples, such as SVF in **Study II and III** which encompasses many different cell types including immune cells, adipocyte progenitor cells, endothelial cells, and many more. Antibody isotype controls are less necessary these days with the reliability of antibody production from reputable sources but can still be useful when detecting lowly expressed markers and when working with cells which highly express Fc receptors to determine the signal associated with nonspecific antibody binding.

These considerations were made during the design of **Study II and Study III** where adipose progenitors (CD45-/CD31-/CD34+), CD4+ T cells (CD45+/CD3+/CD19-/CD4+/CD8-), CD8+ T cells (CD45+/CD3+/CD19-/CD8+/CD4-) M1-ATM (CD45+/CD14+/CD11+/CD206+), and M2-ATM (CD45+/CD14+/CD11-/CD206+) as well as total T cells (CD45+/CD3+/CD19-) and total ATMs (CD45+/CD14+/CD206+) were sorted for further analysis.

3.5.2 Panel design

Each antibody is conjugated to a fluorochrome, a chemical dye which emits light upon excitation by specific light wavelengths. When pairing antibodies and fluorochromes for improved resolution, it is important to consider the expression of the marker on the cells

to be interrogated in addition to the emitted light brightness of the fluorophore, e.g. lowly expressed markers paired with bright fluorophores and vice versa. Typically, fluorophores will be maximally excited by one laser which will result in light emission of a varying range of wavelengths. The detection apparatus will narrow the range to specific wavelength interval which reduces the capacity for spectral overlap. An ideal fluorochrome will incorporate a fluorophore with a long emission wavelength, large Stokes shift, high extinction coefficient, and high quantum efficiency. Even when adjusting for these considerations, spectral overlap will occur, Figure 3.1. Thus, compensation calculation software is necessary which performs a mathematical correction to adjust the cytometric data for the contributions of a given fluorochrome to all other fluorescence channels [129]. Single stain controls on protein coated microparticles are necessary for setting up channel detection measures for compensation calculation; with highly expressed proteins where microparticles are insufficient it becomes necessary to stain individual markers on cells. To control for fluorescence spillover and reduce false positives associated with spectral overlap, fluorescence minus one (FMO) controls are considered the gold standard; these include all fluorochromes except for one to identify the contribution to the signal of all colors in the panel except for the one designed for the given channel. Ideally, FMOs should always be included for all channels in complex panels.

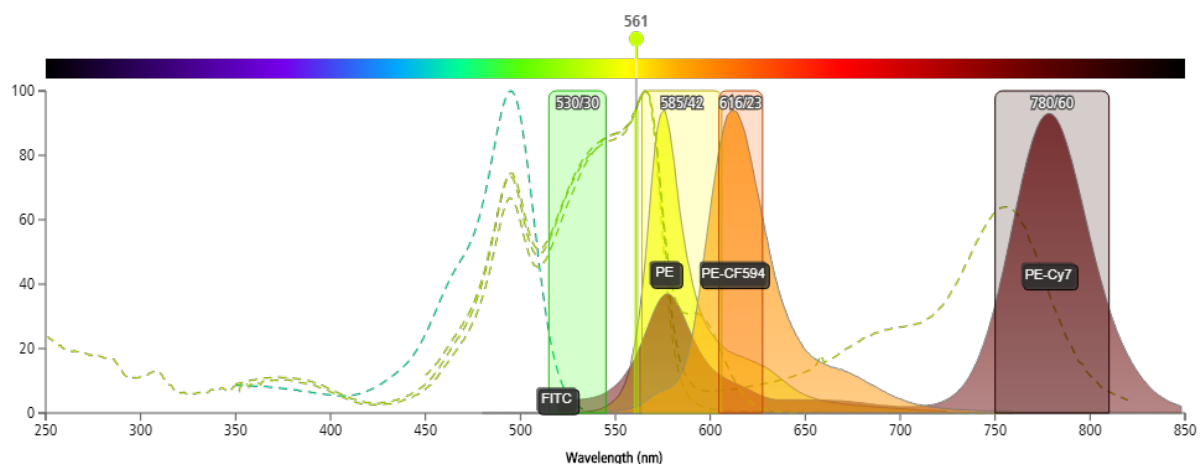


Figure 3.1 Spectrum viewer visualization of the spectral overlap between four fluorochromes used in Study II excited using the 561 nm laser. Created using BD Biosciences Spectrum Viewer tool.

3.6 Clinical metabolic measures

3.6.1 HOMA

The homeostatic model assessment -Insulin Resistance (HOMA-IR) method is a model used to quantify insulin resistance based on a mathematical formula developed by Matthews in 1985 [130]. It is calculated by:

$$\text{HOMA-IR} = \frac{\text{fasting blood glucose} \cdot \text{fasting plasma insulin}}{22.5}$$

As it is based on fasting hormone levels HOMA-IR primarily reflects hepatic insulin resistance. A normal HOMA-IR value is 1 but there is no unanimously accepted cut-off to define insulin resistance. Values up in the range of 1.7-2.0 may be therefore be considered normal, while values above 2.3-2.5 are often defined as insulin resistance [131, 132]. The HOMA model is easy to use as it requires a single fasting plasma sample assayed for insulin and glucose levels. While there are other methods to indirectly estimate insulin sensitivity by fasting plasma measures (e.g. QUICKI, Matsuda etc), HOMA-IR is still widely used and it has proven to be a robust clinical measure in the assessment of diabetes pathophysiology in larger cohorts.

3.6.2 Hyperinsulinemic euglycemic clamps

The patients in **Study II** underwent a hyperinsulinaemic–euglycaemic clamp procedure as described by Hagstrom-Toft et al. [133]. In brief, a bolus dose of insulin was delivered intravenously (1.6 U/m² body surface area) and then an insulin infusion was delivered intravenously (0.12 U/m² min) for 120 min. Every fifth minute, blood glucose values were measured in duplicate and euglycaemia was maintained by a variable intravenous infusion of glucose (50 mg/ml). The measurements from the last 60 min of the clamp were used for calculations of glucose-disposal rate, M-value. During this phase, the insulin levels remained steady (mean ± SD 1,669 ± 528 pmol/l). The hyperinsulinaemic-euglycemic clamp is considered the gold standard for insulin sensitivity assessment. When using high insulin infusion levels (0.12 U/m² min), the M-value primarily reflects insulin sensitivity in skeletal muscle as glucose uptake in the liver is virtually abrogated. To measure hepatic glucose uptake requires lower insulin concentrations and isotope-based tracer techniques. Clamps are labor intensive, time consuming and expensive procedures which are further impacted by patient availability. It is therefore primarily used when the study size is small.

3.7 Ethical approval

These studies were performed in accordance with guidelines set forth by The Declaration of Helsinki and approved by regional ethical committees. All participants received detailed information on the studies. Oral and written informed consent was obtained.

Chapter 4

Results

4.1 Study I: MAFB

In **Study I** we demonstrate that MAFB mRNA expression increases with increased BMI, is enriched in ATM, and that MAFB mediates TNF α effects in adipocytes and THP1 cells used as macrophage model.

Using Cohort 1, we compared non-obese and obese individuals where MAFB expression correlated positively with BMI, (**Study I**, Figure 1). This finding was confirmed in a second cohort where MAFB was also strongly correlated with metabolic syndrome. Multiple regression analysis in this cohort revealed that approximately 60% of the variation in MAFB expression is attributed to both BMI and the metabolic syndrome (**Study I**, table 1). Furthermore, MAFB regulation by adipose mass was established in a third cohort of patients undergoing weight reduction where MAFB expression in scWAT was diminished.

To investigate MAFB association with adipocyte metabolism, insulin-stimulated lipogenesis and isoprenaline-stimulated lipolysis measurements were performed on adipocytes from all subjects of cohort 1. Here we observed negative correlations between scWAT MAFB expression and both stimulated lipogenesis and stimulated lipolysis of isolated adipocytes (**Study I**, Figure 2), which remained significant after adjusting for BMI. Further, MAFB held a correlation to adipocyte size which suggested a role for MAFB in impaired adipocyte biology and possibly hypertrophy, Figure 4.1 (unpublished data).

As MAFB has been reported to be involved in development and differentiation programs in other cell types, we next looked at the impact of MAFB on adipogenesis. We found that MAFB was strongly induced during adipogenesis, but knockdown of MAFB by siRNA had no effect on lipid accumulation, basal lipolysis, basal lipogenesis or stimulated lipogenesis in human adipocytes (**Study I**, Figure 3). However, the TNF α repression of lipid accumulation and upregulated lipolysis rate were abrogated when

MAFB was knocked down indicating that MAFB expression is a requisite factor for TNF α mediated effects on human adipocytes. These results set the stage to investigate a role for MAFB in adipose tissue inflammation.

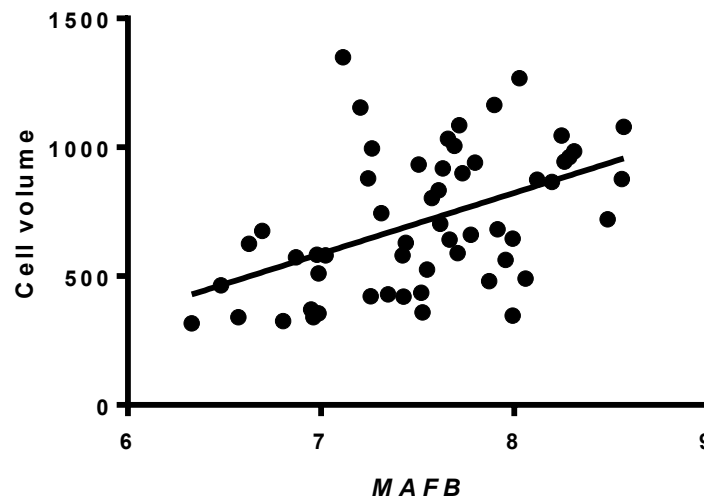


Figure 4.1 MAFB mRNA expression in adipose tissue from cohort 1 correlates with adipocyte hypertrophy ($r=0.47$, $p<0.001$).

The next step was to investigate MAFB regulated inflammation in adipocytes. *In vitro* knockdown of MAFB mRNA alone did not affect expression of the inflammatory genes *CCL2*, *IL6* or *IL8*. However, TNF α , as expected, drastically upregulated the expression of these genes in mature *in vitro* differentiated adipocytes. SiRNA targeting of MAFB yielded no effect on TNF α -induced upregulation of *CCL2* or *IL8*, but the stimulation of *IL6* expression was indeed repressed by approximately 50% (**Study I**, Figure 4). Similar effect was observed on *IL6* protein secretion and thereby demonstrated MAFB involvement in regulation of inflammatory phenotype of adipocytes.

MAFB upregulation by TNF α has been reported in macrophages. Although we could confirm concentration-dependent MAFB upregulation by TNF α in THP1 cells, the effects in adipocytes were inversely regulated (**Study I**, Figure 5). In addition, TNF α mediated effects were present already at 6 hours of stimulation and further pronounced with prolonged stimulation up to 24 hours. To investigate which kinases in the TNF α signaling pathway were involved, adipocytes and THP1 monocytes were treated with kinase inhibitors specific to c-Jun N-terminal kinase (JNK), p44/42, and NF κ B prior to stimulation with TNF α . TNF α downregulation of MAFB in adipocytes was only partially rescued by JNK inhibition whereas both JNK and p44/42 inhibitors significantly suppressed the TNF α induced upregulation of MAFB in monocytes (**Study I**, Figure 5), which is further evidence of differential regulation in the two cell types.

As MAFB is downregulated by pro-inflammatory cytokine TNF α in adipocytes, but upregulated in THP1 monocytes and obesity, we hypothesized that the observation in

obese patients could be due to macrophage infiltration or phenotypical change due to inflamed adipose environment. To assess this, scWAT macrophages and leukocytes were sorted from scWAT SVF and gene expression was compared with paired adipocytes from 6 patients which revealed that macrophages are major cell type expressing MAFB (**Study I**, Figure 6). In addition, there was no difference in MAFB expression in adipocytes from lean and obese patients. Furthermore, MAFB expression strongly correlated with CD14 (monocyte/macrophage marker) expression in WAT.

4.2 Study II: Increased fat cell size

In **Study II**, we observed that non-obese men with type 2 diabetes (n=14) had significantly larger fat cells than healthy subjects (n=13) matched for gender, age, BMI and total fat mass. Diabetic subjects differed from healthy subjects only in clinical measures expected of the diagnosis, e.g. HbA_{1c}, plasma glucose, plasma insulin, HOMA-IR, and M-value. Glycerol release and secretion of inflammatory proteins did not differ between the groups (**Study II**, Table 1). In the entire cohort fat cell size correlated positively with HOMA-IR, glycerol release, TNF α secretion. Further, TNF α secretion correlated with glycerol release and HOMA-IR (**Study II**, Figure 1).

Next we analyzed adipose stromal cells from this cohort by flow cytometry. Population frequencies were normalized to the total number of viable cells (**Study II**, Table 2) and did not differ between the groups. Although the average frequency of M1 ATM was twice as high and the ration of T cells to B cells was approximately 1.5 as high in diabetic patients compared to healthy controls, these values were not significantly different due to large inter-individual variation (visualized in **Study II**, Figure 2). This may also be reflective of the small sample size. While a larger cohort for this characterization would be ideal it is not feasible.

Given a seeming tendency of macrophages to be approximately doubled in diabetes subjects compared to healthy controls, we were interested if there was any macrophage association with fat cell size across the entire cohort. We observed a positive correlation between the M1/M2 ATM ratio and fat cell size. In addition, this ratio positively correlated with both TNF α secretion, and glycerol release (**Study II**, Figure 3).

Genes involved in cell commitment (*BMP4*, *DKK1*, and *WISP2*) and regulation of adipogenesis (*CEBPA*, *PPARG*, *EBF1*, and *SLC2A4*) were quantified in adipose progenitors or intact tissue, respectively. *BMP4* in progenitor cells correlated negatively with fat cell size (**Study II**, Figure 4). *DKK1* (negative) and *WISP2* (positive adipogenesis regulators) exhibited near significant trends in relation to fat cell size. In adipose tissue, *CEBPA*, *PPARG*, *EBF1* and *SLC2A4* mRNA exhibited strong negative correlations with fat cell size (**Study II**, Figure 5).

4.3 Study III: Single-cell transcriptomics

The transcriptomes of 574 individual cells from four healthy women were analyzed by clustering and visualized through t-distributed stochastic neighbor embedding (**Study III**, Figure 1a-b). Four distinct groups of cells were identified by a first level clustering of SVF by cell and gene expression based on 2000 most variable genes. Each cluster was evident in all four individuals, and further evident by t-SNE projection. We identified the genes that best characterized each cluster and mapped them back to microarray data from FACS sorted SVF of scWAT (**Study III**, Figure 1c-d). We found that the largest fraction was indeed adipose progenitor cells while the 3 minor fractions were ATM or M1-like, M2-like and an intermediate phenotype. Key marker genes of each cell type were projected onto the t-SNE plot to confirm the predicted populations (**Study III**, Figure 1). Interestingly, only macrophages demonstrated sub-clusters, adipose progenitor transcriptomes did not.

To further investigate the possibility that adipose progenitors include some additional subtypes, a second level clustering was performed in which most variable genes within progenitor cluster were identified and then cell and gene clustering was performed and visualized by heatmap (**Study III**, Figure 2a). However, no subtypes appeared evident. Additionally, the transcriptomes were assessed for rare cells but this too yielded no distinct subtypes. In light of no clear sub-clusters of adipose progenitor cells, we evaluated a hypothesis that cells might be discernable by stage of differentiation. To this end, we placed cells along a pseudotemporal ordering in t-SNE projection and screened for pseudotime-dependent genes (**Study III**, Figure 2b). Seventy such genes over the significance cutoff point were found. Collagen genes, CD55 and Thy1 were among the most highly expressed genes in the beginning of pseudotime while ribosomal genes and KLF4 were elevated towards the end of the pseudotemporal space (**Study III**, Figure 2c). While we were able to separate adipose progenitor cells into four pseudotime-dependent states, amplitude differences in gene expression between the states were very minor. Furthermore we were unable to distinguish between the states when projected on t-SNE plot of the entire data set. Thus we found no evidence that adipose progenitors constitute a heterogeneous population in healthy human adipose tissue.

Chapter 5

Discussion

In **Study I**, we observed an increase in MAFB expression in obese adipose tissue. MAFB expression is enriched in macrophages whereby it is differentially regulated in macrophages and adipocytes. Further, we demonstrated that MAFB mediates TNF α -induced inflammation in adipocytes.

MAFB has been shown to regulate differentiation programs in a variety of cell types [106-109]. Our study showed that MAFB did not directly influence the adipogenesis process in human adipocytes as it did not affect lipid accumulation when MAFB was knocked down before induction of differentiation. However, MAFB expression was strongly induced by adipocyte differentiation which implied the possibility of its role in metabolism in the mature adipocyte. Its upregulation during adipogenesis could be in part due to regulation by CEPBD, as similarly reported in keratinocytes [106]. As MAFB expression correlated with fat cell size, it is feasible that Wnt-signaling in conjunction with the early adipogenic transcriptional regulator CEPBD work in concert to mediate hypertrophy.

MAFB expression was differentially regulated by TNF α in adipocytes (suppressed) versus macrophages (induced), in addition it strongly correlated with CD14 (**Study I**, Fig 6C) and CD68 (data not shown), markers of adipose tissue macrophages. Thus, we draw the conclusion that MAFB is a good marker of macrophage abundance in WAT. Therefore, the elevated MAFB mRNA levels which correlate with increased adiposity (BMI) are likely due to increased macrophage content in WAT. Alternatively, MAFB can also show the increased pro-inflammatory phenotype of adipose tissue as its expression in macrophages is induced by TNF α . So even though MAFB is expressed in adipocytes, it is likely that in intact WAT the contribution by macrophages outweighs adipocyte expression and MAFB mRNA correlations with clinical measures are likely driven by macrophages.

The major role of MAFB in adipocytes appears to be as a mediator of inflammation. MAFB seems to be required for TNF α signaling mediating upregulation of some

inflammatory genes (IL6) (**Study I**, Fig 4). Interestingly, MAFB is downregulated by TNF, but is required for TNF signaling, which could suggest some compensatory protective feedback loop to keep inflammation at low levels. One could speculate that further therapeutic downregulation of MAFB in adipocytes could be protective against obesity-induced WAT inflammation. However, the mechanism how MAFB interacts with protein components of TNF α signaling is unknown.

In **Study II**, we analyzed adipose tissue metabolism, morphology and stromal cells isolated from scWAT biopsies in a clinically characterized cohort of non-obese men with and without type 2 diabetes by fluorescence activated cell sorting followed by gene expression studies.

Although type 2 diabetic patients differed from healthy subjects in the expected clinical measures, there were no differences in lipolysis or inflammatory protein secretion between the two groups. Therefore, one could speculate that these measures are closer linked to obesity than type 2 diabetes. Adipocyte hypertrophy was the principal factor differentiating type 2 diabetes subjects from healthy individuals. This suggests that adipocyte hypertrophy is an important phenotype linking WAT to type 2 diabetes. In line with this, we found correlations between fat cell size and HOMA-IR, glycerol secretion, and TNF α secretion. We, therefore, identified fat cell size as a phenotype worth further investigation. Previous studies showed that macrophage abundance is elevated in unhealthy adipose tissue in subjects with or without the metabolic syndrome [52]. However, upon 13-color analysis and sorting by flow cytometry, we further could not separate the two groups as there were no significant differences in SVF cell population frequencies between the groups. This was a surprising finding given the elevated inflammation typically associated with insulin resistance and the metabolic syndrome in adipose tissue. However, we noticed that macrophage frequencies appeared to be doubled in type 2 diabetes subjects but the difference was not significant due to large inter-individual variation and the small cohort size, even though the size is similar to comparable studies [134-136]. In addition, we observed correlations between fat cell size and the M1/M2 ATM ratio and this ratio also correlated with basal lipolysis as well as TNF α secretion. This evidence is in line with findings from **Study I** owing, at least in part, adverse metabolic and inflammatory phenotypes in adipose tissue to the presence of pro-inflammatory macrophages.

Which molecular mechanisms are at play in adipocyte hypertrophy? Previous studies by the Smith laboratory suggested defective adipogenesis linked to WNT-signaling [67-70, 137, 138]. It has been shown that BMP4 plays a critical role early in the adipogenesis program by dissociating WISP2 (a Wnt-signaling molecule and negative regulator of adipogenesis) from the PPAR γ transcriptional activator ZNF423 (a positive regulator of adipogenesis). This allows ZNF423 to relocate to the nucleus to initiate adipogenesis. Our observation of a negative correlation between adipocyte fate and adipogenesis regulating

genes and fat cell size in WAT are in line with diminished capacity of WAT to produce new fat cells in hypertrophy.

Taken together we find that non-obese diabetic subjects have larger fat cells than their healthy counterparts and most likely exhibit an impaired adipogenesis program. Adipose tissue hypertrophy rather than specifically diabetic status is an indicator of a reduced metabolic capacity and impaired insulin sensitivity in scWAT.

In **Study III**, we investigated the possible heterogeneity in adipocyte progenitor cells by single cell mRNA sequencing of total resident SVF in WAT as has been reported in murine and human studies [139-142]. Some studies show that scWAT derived progenitor cells are more enriched in adipogenic transcriptional regulators than progenitor cells in visceral adipose depots in human [143-146]. Further, scWAT progenitor cells express higher levels of adipogenic transcription factors during adipogenesis than visceral derived cells [145]. Given the findings in **Study II** in addition to the aforementioned previous reports, we hypothesized that adipocyte progenitor cells might have multiple activation states or subtypes which predispose hypertrophy, or hyperplasia, in WAT which could explain why these states differ between patients with varying adiposity and metabolic status. Contrary to our hypothesis, the principle finding is that, at least in healthy individuals, human adipocyte progenitor cells constitute a single, homogeneous population.

A pseudotemporal ordering of revealed a gradient expression of 70 genes deemed significant. Along the pseudotime, typical markers of adipogenic capacity (CD34, CD36, PDGFRA) were not present, where other known markers (CD55, SFRP4, SEMA3C) were found to be significantly regulated. Unfortunately, further functional studies of the latter genes proved limiting as differences in expression levels were small and these genes were not labeling distinct populations when labeled on TSNE plots. While CD34, CD36 and PDGFRA were not among the pseudotime gradient of genes, they were certainly detected and thereby not missed in the preparation of SVF or subsequent treatments of the samples. While our results show a single adipose progenitor population in scWAT of healthy individuals, we cannot exclude a possibility of subtypes in subjects exhibiting obesity, insulin resistance or even within other adipose depots. Furthermore, an inherent weakness of our study is indeed the use of the Fluidigm C1 system as opposed to other methods, including single cell FACS sorting following my mRNA sequencing. The C1 microfluidic chip used for this study was designed to capture cells within a specific diameter range, 10-17 μm , which excludes the smaller and larger cell not captured by chance. Thus, there is a possibility that some progenitor subtypes were missed although our measures did calculate a majority of progenitor cell diameters to fall within this range. Additionally, this study assessed heterogeneity from the mRNA level and found no evidence of subtypes. If heterogeneity is detectable by mRNA levels then it could possibly require sequencing of many more single cells than were feasible at the time the

study was performed. However, this cannot exclude the possibility that heterogeneity is not evident by mRNA analyses alone and must be complemented with other analyses, e.g. epigenetics or gene-specific approaches. Furthermore, it is critically important to keep in context that studies which show heterogeneity in adipose progenitor cells have been performed in murine models and those findings may owe to species differences [71, 72]. This study has an inherent strength in that it performed single-cell RNA sequencing of more than 33,000 stromal cells from two murine adipose depots under *in vivo* control and treatment conditions [72]. This is more than a 50-fold difference relative to **Study III**. However, such a study is very difficult to perform in humans given patient specific differences compared to single genetic background that is most often used in murine studies.

Furthermore, an interesting finding was observed in relation to **Studies I and II**, multiple macrophage clusters. The literature has long been at odds with the widely-used M1-M2 macrophage nomenclature insisting that it was too simplistic a characterization of a complex cell type. Furthermore these assessments were based on *in vitro* polarization programs, which are not always reflective of the *in vivo* state. Our data show that there are at least 3 macrophage subtypes in scWAT of healthy subjects which we called M1-like, M2-like and, interestingly, and intermediate phenotype, which could possibly have a role independent of inflammation. We should also mention that the expression of macrophage populations-specific genes defined by single-cell sequencing was investigated in FACS-sorted SVF fractions where M1 macrophages were defined as CD45+/CD14+/CD206+/CD11c+ cells and M2 as CD11c- cells. Therefore, it would be more correct to say that one cluster of cells mapped to CD11c+ and the other to CD11c- population and the third one mapped to both. The exact phenotypes and possible origins of the cells belonging to these clusters in addition to functional characterization remain to be determined in future investigations.

Finally, although the ability of our analysis to separate macrophage subtypes validates the approach in general, it failed to reveal heterogeneity of adipose progenitor cells meaning that these cells most likely exhibit very minor differences compared to macrophage cell populations. Though, our study was limited by study size, transcriptomes analyzed, and a lack of disease states for comparison it was the first of its kind in human adipose tissue.

Chapter 6

Concluding Remarks and Future Perspectives

This thesis aimed to study inflammation and adipogenesis capacity in human subcutaneous white adipose tissue with respect to the development of obesity and associated comorbidities, including insulin resistance. What we found was elevated expression of MAFB, a now important mediator of adipose tissue inflammation, with respect to BMI, increased adipocyte hypertrophy, and a shift in macrophage phenotypes in relation to insulin resistance. Furthermore, we found that in healthy subjects the age-old question of what causes hypertrophic or hyperplastic adipose tissue cannot be easily addressed looking in search for distinct populations of progenitor cells, Figure 6.1. In addition, our data confer a result which the macrophage world has long considered, multiple macrophage subtypes; this is a concept which exceeds the bounds of the classical (M1) vs alternative (M2) activation polarization paradigm.

Interestingly, MAFB did not seem to mediate metabolic consequences on its own although it was certainly expressed throughout the adipogenesis program. It can be speculated that MAFB functions through CEBPD to affect adipocyte size indirectly. Now established as an interesting marker of adipose tissue inflammation, there is potential for MAFB to serve as a therapeutic target in adipocytes specifically as MAFB mRNA disturbances could lead to improved adipogenic capacity by way of a reduced TNF α -signaling capacity.

Adipocyte hypertrophy was the focal point of **Study II** and the initiator of **Study III**. We found that adipocyte size corresponded adverse metabolic clinical phenotypes as well as inflammatory macrophage content. Addressing this issue would be of key clinical emphasis where one avenue could be through the improvement of the thiazolidinediones class of drugs which activate PPAR γ and thereby improve adipogenic capacity. One such drug used to treat patients with type 2 diabetes is rosiglitazone which had been removed from the market due to elevated risk of myocardial infarction and death [148]. However,

recent meta-analyses confirm [149] or question [150] the significance of these findings. In either case, more investigation is needed into this class of anti-diabetic drugs. These three studies cemented a role for macrophages in the forefront of adipose research by confirming inflammatory and metabolic roles but also stoking the flames of a paradigm shift in macrophage classification. Study II utilized established adipose tissue macrophage markers whereby mixed phenotypes had been reported for cells expressing markers of classical or alternative activation [54, 77]. Study III supported this notion of multiple phenotypes, or even a possibility of varied macrophage origin [83-85], by reporting at least three ATM subtypes.

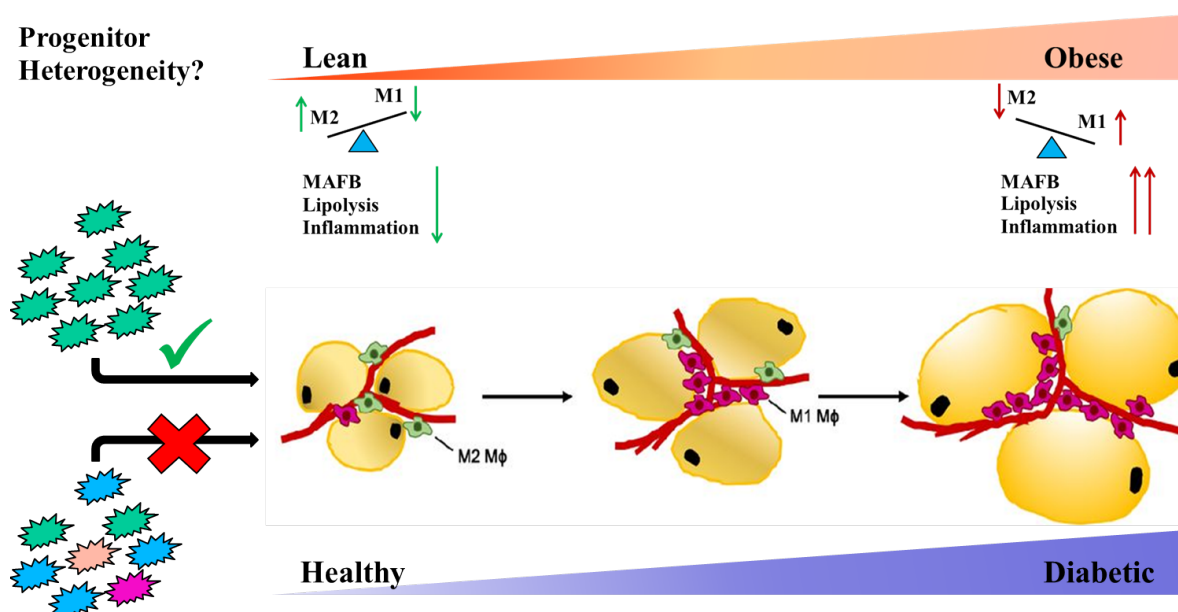


Figure 6.1 Central findings in this thesis. Adipose progenitor cells consist of one homogeneous population. Dysregulated adipogenesis leads to hypertrophic adipose tissue during obesogenic conditions which is further characterized by increased rates of basal lipolysis. Macrophage populations become imbalanced leading to a pro-inflammatory microenvironment. Image adapted, in part, from Chan et. al [147]. DOI: 10.1042/CS20180041

Indeed, there is much more work to be done. It is imperative to this field of research to follow-up these studies and investigate differences between progenitors of healthy subjects with subjects displaying clinically relevant conditions, e.g. obesity, insulin resistance, dyslipidemia, and type 2 diabetes. Many more cells from larger cohorts would be needed for such an endeavor. It would take decades to acquire patient samples to answer critical questions to ascertain the impact of disease state, BMI status, weight loss effects on adipose biology, and inflammation on adipogenesis and adipose composition. Such a study would benefit from paired human adipose biopsies from both scWAT and visceral WAT. Further, it would require skilled bioinformatics analysts to separate technical noise from biologically relevant output, to combine sequencing, proteomics and

flow cytometric data from the same cohorts of individuals as well as state-of-art techniques of functional analysis. Such investigations are indeed ambitious endeavors; however, they provide opportunity for major advancement in the field of adipose biology. While the monetary investment would undoubtedly be high, the potential for curbing the progression of metabolic diseases is arguably worthwhile.

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References

1. Hoffmans, M.D., D. Kromhout, and C. de Lezenne Coulander, *The impact of body mass index of 78,612 18-year old Dutch men on 32-year mortality from all causes*. J Clin Epidemiol, 1988. **41**(8): p. 749-56.
2. James, P.T., et al., *The worldwide obesity epidemic*. Obes Res, 2001. **9 Suppl 4**: p. 228S-233S.
3. James, P.T., N. Rigby, and R. Leach, *The obesity epidemic, metabolic syndrome and future prevention strategies*. Eur J Cardiovasc Prev Rehabil, 2004. **11**(1): p. 3-8.
4. Padwal, R.S. and A.M. Sharma, *Prevention of cardiovascular disease: obesity, diabetes and the metabolic syndrome*. Can J Cardiol, 2010. **26 Suppl C**: p. 18C-20C.
5. Gallagher, E.J. and D. LeRoith, *Obesity and Diabetes: The Increased Risk of Cancer and Cancer-Related Mortality*. Physiol Rev, 2015. **95**(3): p. 727-48.
6. Kiliaan, A.J., I.A. Arnoldussen, and D.R. Gustafson, *Adipokines: a link between obesity and dementia?* Lancet Neurol, 2014. **13**(9): p. 913-23.
7. Reaven, G.M., *Relationships among insulin resistance, type 2 diabetes, essential hypertension, and cardiovascular disease: similarities and differences*. J Clin Hypertens (Greenwich), 2011. **13**(4): p. 238-43.
8. Milic, S., D. Lulic, and D. Stimac, *Non-alcoholic fatty liver disease and obesity: biochemical, metabolic and clinical presentations*. World J Gastroenterol, 2014. **20**(28): p. 9330-7.
9. Lee, B.C. and J. Lee, *Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance*. Biochim Biophys Acta, 2014. **1842**(3): p. 446-62.
10. Ong, W.K. and S. Sugii, *Adipose-derived stem cells: fatty potentials for therapy*. Int J Biochem Cell Biol, 2013. **45**(6): p. 1083-6.
11. O'Rourke, R.W., et al., *Hypoxia-induced inflammatory cytokine secretion in human adipose tissue stromovascular cells*. Diabetologia, 2011. **54**(6): p. 1480-90.
12. Hotamisligil, G.S., et al., *Tumor necrosis factor alpha inhibits signaling from the insulin receptor*. Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4854-8.
13. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance*. Science, 1993. **259**(5091): p. 87-91.
14. Szalkowski, D., et al., *Antidiabetic thiazolidinediones block the inhibitory effect of tumor necrosis factor-alpha on differentiation, insulin-stimulated glucose uptake, and gene expression in 3T3-L1 cells*. Endocrinology, 1995. **136**(4): p. 1474-81.

15. Zhang, H.H., et al., *Tumor necrosis factor-alpha stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP*. Diabetes, 2002. **51**(10): p. 2929-35.
16. Zhang, K., et al., *Adiponectin Suppresses T Helper 17 Cell Differentiation and Limits Autoimmune CNS Inflammation via the SIRT1/PPARgamma/RORgamma Pathway*. Mol Neurobiol, 2017. **54**(7): p. 4908-4920.
17. Nicholson, T., et al., *The role of adipokines in skeletal muscle inflammation and insulin sensitivity*. J Inflamm (Lond), 2018. **15**: p. 9.
18. Unamuno, X., et al., *Adipokine dysregulation and adipose tissue inflammation in human obesity*. Eur J Clin Invest, 2018: p. e12997.
19. Ohashi, K., et al., *Role of anti-inflammatory adipokines in obesity-related diseases*. Trends Endocrinol Metab, 2014. **25**(7): p. 348-55.
20. Ouchi, N., et al., *Adipocytokines and obesity-linked disorders*. Nagoya J Med Sci, 2012. **74**(1-2): p. 19-30.
21. Weyer, C., et al., *Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts Type II diabetes independent of insulin resistance*. Diabetologia, 2000. **43**(12): p. 1498-1506.
22. Yang, J., et al., *The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients*. Obesity (Silver Spring), 2012. **20**(5): p. 932-8.
23. Salans, L.B., J.L. Knittle, and J. Hirsch, *The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity*. J Clin Invest, 1968. **47**(1): p. 153-65.
24. Arner, P., *Human fat cell lipolysis: biochemistry, regulation and clinical role*. Best Pract Res Clin Endocrinol Metab, 2005. **19**(4): p. 471-82.
25. Kolditz, C.I. and D. Langin, *Adipose tissue lipolysis*. Curr Opin Clin Nutr Metab Care, 2010. **13**(4): p. 377-81.
26. Jenkins, C.M., et al., *Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities*. J Biol Chem, 2004. **279**(47): p. 48968-75.
27. Zechner, R., et al., *FAT SIGNALS - Lipases and Lipolysis in Lipid Metabolism and Signaling*. Cell Metab, 2012. **15**(3): p. 279-291.
28. Holm, C., et al., *Hormone-sensitive lipase: sequence, expression, and chromosomal localization to 19 cent-q13.3*. Science, 1988. **241**(4872): p. 1503-6.
29. Lindvall, H., et al., *A novel hormone-sensitive lipase isoform expressed in pancreatic beta-cells*. J Biol Chem, 2004. **279**(5): p. 3828-36.
30. Wang, H., et al., *Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins*. J Biol Chem, 2009. **284**(46): p. 32116-25.
31. Osuga, J., et al., *Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity*. Proc Natl Acad Sci U S A, 2000. **97**(2): p. 787-92.
32. Zimmermann, R., et al., *Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase*. Science, 2004. **306**(5700): p. 1383-6.

33. Granneman, J.G., et al., *Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl)*. J Biol Chem, 2009. **284**(50): p. 34538-44.
34. Galic, S., J.S. Oakhill, and G.R. Steinberg, *Adipose tissue as an endocrine organ*. Mol Cell Endocrinol, 2010. **316**(2): p. 129-39.
35. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease*. Nat Rev Immunol, 2011. **11**(2): p. 85-97.
36. Barcellini, W., et al., *TH1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients*. AIDS, 1994. **8**(6): p. 757-62.
37. Fain, J.N., et al., *Comparison of PGE2, prostacyclin and leptin release by human adipocytes versus explants of adipose tissue in primary culture*. Prostaglandins Leukot Essent Fatty Acids, 2002. **67**(6): p. 467-73.
38. Fain, J.N., et al., *Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans*. Endocrinology, 2004. **145**(5): p. 2273-82.
39. Madan, A.K., et al., *Comparison of IL-8, IL-6 and PGE(2) formation by visceral (omental) adipose tissue of obese Caucasian compared to African-American women*. Obes Surg, 2006. **16**(10): p. 1342-50.
40. Mozaffarian, D., et al., *Heart disease and stroke statistics--2015 update: a report from the American Heart Association*. Circulation, 2015. **131**(4): p. e29-322.
41. Bluher, M. and C.S. Mantzoros, *From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century*. Metabolism, 2015. **64**(1): p. 131-45.
42. Lehr, S., S. Hartwig, and H. Sell, *Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders*. Proteomics Clin Appl, 2012. **6**(1-2): p. 91-101.
43. Rosen, E.D. and B.M. Spiegelman, *What we talk about when we talk about fat*. Cell, 2014. **156**(1-2): p. 20-44.
44. Bjorntorp, P., *Sjostrom L, +SJOSTROM L: Number and size of adipose tissue fat cells in relation to metabolism in human obesity*. Metabolism, 1971. **20**(7): p. 703-13.
45. Bjorntorp, P., *Effects of age, sex, and clinical conditions on adipose tissue cellularity in man*. Metabolism, 1974. **23**(11): p. 1091-102.
46. Bjorntorp, P., A. Gustafson, and B. Persson, *Adipose tissue fat cell size and number in relation to metabolism in endogenous hypertriglyceridemia*. Acta Med Scand, 1971. **190**(5): p. 363-7.
47. Acosta, J.R., et al., *Increased fat cell size: a major phenotype of subcutaneous white adipose tissue in non-obese individuals with type 2 diabetes*. Diabetologia, 2016. **59**(3): p. 560-70.
48. Kahn, S.E., R.L. Hull, and K.M. Utzschneider, *Mechanisms linking obesity to insulin resistance and type 2 diabetes*. Nature, 2006. **444**(7121): p. 840-6.
49. Gustafson, B., et al., *Inflammation and impaired adipogenesis in hypertrophic obesity in man*. Am J Physiol Endocrinol Metab, 2009. **297**(5): p. E999-E1003.
50. DeFuria, J., et al., *B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile*. Proc Natl Acad Sci U S A, 2013. **110**(13): p. 5133-8.

51. Stefanovic-Racic, M., et al., *Dendritic cells promote macrophage infiltration and comprise a substantial proportion of obesity-associated increases in CD11c+ cells in adipose tissue and liver*. Diabetes, 2012. **61**(9): p. 2330-9.
52. Esser, N., et al., *Obesity phenotype is related to NLRP3 inflammasome activity and immunological profile of visceral adipose tissue*. Diabetologia, 2013. **56**(11): p. 2487-97.
53. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue*. J Clin Invest, 2003. **112**(12): p. 1796-808.
54. Wentworth, J.M., et al., *Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity*. Diabetes, 2010. **59**(7): p. 1648-56.
55. Arner, E., M. Ryden, and P. Arner, *Tumor necrosis factor alpha and regulation of adipose tissue*. N Engl J Med, 2010. **362**(12): p. 1151-3.
56. Glass, C.K. and J.M. Olefsky, *Inflammation and lipid signaling in the etiology of insulin resistance*. Cell Metab, 2012. **15**(5): p. 635-45.
57. Olefsky, J.M. and C.K. Glass, *Macrophages, inflammation, and insulin resistance*. Annu Rev Physiol, 2010. **72**: p. 219-46.
58. Verdeguer, F. and M. Aouadi, *Macrophage heterogeneity and energy metabolism*. Exp Cell Res, 2017. **360**(1): p. 35-40.
59. Kang, H.S., et al., *Advanced Properties of Urine Derived Stem Cells Compared to Adipose Tissue Derived Stem Cells in Terms of Cell Proliferation, Immune Modulation and Multi Differentiation*. J Korean Med Sci, 2015. **30**(12): p. 1764-76.
60. Arner, P., et al., *Dynamics of human adipose lipid turnover in health and metabolic disease*. Nature, 2011. **478**(7367): p. 110-3.
61. Spalding, K.L., et al., *Dynamics of fat cell turnover in humans*. Nature, 2008. **453**(7196): p. 783-7.
62. Joe, A.W., et al., *Depot-specific differences in adipogenic progenitor abundance and proliferative response to high-fat diet*. Stem Cells, 2009. **27**(10): p. 2563-70.
63. Kwok, K.H., K.S. Lam, and A. Xu, *Heterogeneity of white adipose tissue: molecular basis and clinical implications*. Exp Mol Med, 2016. **48**: p. e215.
64. Berry, R., E. Jeffery, and M.S. Rodeheffer, *Weighing in on adipocyte precursors*. Cell Metab, 2014. **19**(1): p. 8-20.
65. Arner, P., et al., *Genetic predisposition for Type 2 diabetes, but not for overweight/obesity, is associated with a restricted adipogenesis*. PLoS One, 2011. **6**(4): p. e18284.
66. Lonn, M., et al., *Adipocyte size predicts incidence of type 2 diabetes in women*. FASEB J, 2010. **24**(1): p. 326-31.
67. Gustafson, B., et al., *Restricted adipogenesis in hypertrophic obesity: the role of WISP2, WNT, and BMP4*. Diabetes, 2013. **62**(9): p. 2997-3004.
68. Gustafson, B., et al., *BMP4 and BMP Antagonists Regulate Human White and Beige Adipogenesis*. Diabetes, 2015. **64**(5): p. 1670-81.
69. Gustafson, B., et al., *Insulin resistance and impaired adipogenesis*. Trends Endocrinol Metab, 2015. **26**(4): p. 193-200.

70. Gustafson, B. and U. Smith, *The WNT inhibitor Dickkopf 1 and bone morphogenetic protein 4 rescue adipogenesis in hypertrophic obesity in humans*. Diabetes, 2012. **61**(5): p. 1217-24.
71. Schwalie, P.C., et al., *A stromal cell population that inhibits adipogenesis in mammalian fat depots*. Nature, 2018. **559**(7712): p. 103-108.
72. Burl, R.B., et al., *Deconstructing Adipogenesis Induced by beta3-Adrenergic Receptor Activation with Single-Cell Expression Profiling*. Cell Metab, 2018. **28**(2): p. 300-309 e4.
73. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions*. Immunity, 2010. **32**(5): p. 593-604.
74. Mantovani, A., et al., *Macrophage plasticity and polarization in tissue repair and remodelling*. J Pathol, 2013. **229**(2): p. 176-85.
75. Zeyda, M., et al., *Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production*. Int J Obes (Lond), 2007. **31**(9): p. 1420-8.
76. Kanneganti, T.D. and V.D. Dixit, *Immunological complications of obesity*. Nat Immunol, 2012. **13**(8): p. 707-12.
77. Kratz, M., et al., *Metabolic dysfunction drives a mechanistically distinct proinflammatory phenotype in adipose tissue macrophages*. Cell Metab, 2014. **20**(4): p. 614-25.
78. Kanda, H., et al., *MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity*. J Clin Invest, 2006. **116**(6): p. 1494-505.
79. Morinaga, H., et al., *Characterization of distinct subpopulations of hepatic macrophages in HFD/obese mice*. Diabetes, 2015. **64**(4): p. 1120-30.
80. Amano, S.U., et al., *Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation*. Cell Metab, 2014. **19**(1): p. 162-171.
81. Robbins, C.S., et al., *Local proliferation dominates lesional macrophage accumulation in atherosclerosis*. Nat Med, 2013. **19**(9): p. 1166-72.
82. Zheng, C., et al., *Local proliferation initiates macrophage accumulation in adipose tissue during obesity*. Cell Death Dis, 2016. **7**: p. e2167.
83. Gomez Perdiguero, E., et al., *Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors*. Nature, 2015. **518**(7540): p. 547-51.
84. Jager, J., M. Aparicio-Vergara, and M. Aouadi, *Liver innate immune cells and insulin resistance: the multiple facets of Kupffer cells*. J Intern Med, 2016. **280**(2): p. 209-20.
85. Yosef, N., et al., *The phenotypic and functional properties of mouse yolk-sac-derived embryonic macrophages*. Dev Biol, 2018.
86. Chung, K.J., et al., *Innate immune cells in the adipose tissue*. Rev Endocr Metab Disord, 2018.
87. Nishiyama, K., et al., *Aggressive Crosstalk Between Fatty Acids and Inflammation in Macrophages and Their Influence on Metabolic Homeostasis*. Neurochem Res, 2018. **43**(1): p. 10-17.
88. Xu, X., et al., *Concentration-Dependent Diversification Effects of Free Cholesterol Loading on Macrophage Viability and Polarization*. Cell Physiol Biochem, 2015. **37**(2): p. 419-431.

89. Xu, H., et al., *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance*. J Clin Invest, 2003. **112**(12): p. 1821-30.
90. Wernstedt Asterholm, I., et al., *Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling*. Cell Metab, 2014. **20**(1): p. 103-18.
91. Lago, F., et al., *Adipokines as novel modulators of lipid metabolism*. Trends Biochem Sci, 2009. **34**(10): p. 500-10.
92. Van Gaal, L.F., I.L. Mertens, and C.E. De Block, *Mechanisms linking obesity with cardiovascular disease*. Nature, 2006. **444**(7121): p. 875-80.
93. Arkan, M.C., et al., *IKK-beta links inflammation to obesity-induced insulin resistance*. Nat Med, 2005. **11**(2): p. 191-8.
94. Kataoka, K., et al., *MafB, a new Maf family transcription activator that can associate with Maf and Fos but not with Jun*. Mol Cell Biol, 1994. **14**(11): p. 7581-91.
95. Wang, P.W., et al., *Human KRML (MAFB): cDNA cloning, genomic structure, and evaluation as a candidate tumor suppressor gene in myeloid leukemias*. Genomics, 1999. **59**(3): p. 275-81.
96. Cawthorn, W.P. and J.K. Sethi, *TNF-alpha and adipocyte biology*. FEBS Lett, 2008. **582**(1): p. 117-31.
97. Ryden, M. and P. Arner, *Tumour necrosis factor-alpha in human adipose tissue -- from signalling mechanisms to clinical implications*. J Intern Med, 2007. **262**(4): p. 431-8.
98. MacEwan, D.J., *TNF receptor subtype signalling: differences and cellular consequences*. Cell Signal, 2002. **14**(6): p. 477-92.
99. Hotamisligil, G.S., et al., *Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance*. J Clin Invest, 1995. **95**(5): p. 2409-15.
100. Uysal, K.T., et al., *Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function*. Nature, 1997. **389**(6651): p. 610-4.
101. Fain, J.N., S.W. Bahouth, and A.K. Madan, *TNFalpha release by the nonfat cells of human adipose tissue*. Int J Obes Relat Metab Disord, 2004. **28**(4): p. 616-22.
102. Sartipy, P. and D.J. Loskutoff, *Monocyte chemoattractant protein 1 in obesity and insulin resistance*. Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7265-70.
103. Bruun, J.M., et al., *Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT*. J Clin Endocrinol Metab, 2005. **90**(4): p. 2282-9.
104. Christiansen, T., B. Richelsen, and J.M. Bruun, *Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects*. Int J Obes (Lond), 2005. **29**(1): p. 146-50.
105. Dahlman, I., et al., *A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects*. J Clin Endocrinol Metab, 2005. **90**(10): p. 5834-40.
106. Borrelli, S., et al., *C/EBPdelta gene targets in human keratinocytes*. PLoS One, 2010. **5**(11): p. e13789.
107. Gemelli, C., et al., *MafB is a downstream target of the IL-10/STAT3 signaling pathway, involved in the regulation of macrophage de-activation*. Biochim Biophys Acta, 2014. **1843**(5): p. 955-64.

108. Smink, J.J., et al., *Transcription factor C/EBPbeta isoform ratio regulates osteoclastogenesis through MafB*. EMBO J, 2009. **28**(12): p. 1769-81.
109. Smink, J.J., P.U. Tunn, and A. Leutz, *Rapamycin inhibits osteoclast formation in giant cell tumor of bone through the C/EBPbeta - MafB axis*. J Mol Med (Berl), 2012. **90**(1): p. 25-30.
110. Zanicco-Marani, T., et al., *TFE3 transcription factor regulates the expression of MAFB during macrophage differentiation*. Exp Cell Res, 2009. **315**(11): p. 1798-808.
111. Zhang, Y., Q. Chen, and A.C. Ross, *Retinoic acid and tumor necrosis factor-alpha induced monocytic cell gene expression is regulated in part by induction of transcription factor MafB*. Exp Cell Res, 2012. **318**(18): p. 2407-16.
112. Artner, I., et al., *MafB is required for islet beta cell maturation*. Proc Natl Acad Sci U S A, 2007. **104**(10): p. 3853-8.
113. Artner, I., et al., *MafA and MafB regulate genes critical to beta-cells in a unique temporal manner*. Diabetes, 2010. **59**(10): p. 2530-9.
114. Artner, I., et al., *MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells*. Diabetes, 2006. **55**(2): p. 297-304.
115. Matsuoka, T.A., et al., *Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells*. Mol Cell Biol, 2003. **23**(17): p. 6049-62.
116. Zhao, L., et al., *The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription*. J Biol Chem, 2005. **280**(12): p. 11887-94.
117. Rodbell, M., *METABOLISM OF ISOLATED FAT CELLS. I. EFFECTS OF HORMONES ON GLUCOSE METABOLISM AND LIPOLYSIS*. J Biol Chem, 1964. **239**: p. 375-80.
118. van Harmelen, V., T. Skurk, and H. Hauner, *Primary culture and differentiation of human adipocyte precursor cells*. Methods Mol Med, 2005. **107**: p. 125-35.
119. Dicker, A., et al., *Functional studies of mesenchymal stem cells derived from adult human adipose tissue*. Exp Cell Res, 2005. **308**(2): p. 283-90.
120. Rodriguez, A.M., et al., *Adipocyte differentiation of multipotent cells established from human adipose tissue*. Biochem Biophys Res Commun, 2004. **315**(2): p. 255-63.
121. Pettersson, A.M., et al., *LXR is a negative regulator of glucose uptake in human adipocytes*. Diabetologia, 2013. **56**(9): p. 2044-54.
122. Ehrlund, A., et al., *Characterization of the Wnt inhibitors secreted frizzled-related proteins (SFRPs) in human adipose tissue*. J Clin Endocrinol Metab, 2013. **98**(3): p. E503-8.
123. Littlefield, M.J.T.V., I; Reiss, A.B., *Polarization of Human THP-1 Macrophages: Link between Adenosine Receptors, Inflammation and Lipid Accumulation*. International Journal of Immunology and Immunotherapy, 2014. **1**(1): p. 2-8.
124. Genin, M., et al., *M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide*. BMC Cancer, 2015. **15**: p. 577.
125. Rotondo, F., et al., *Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycero genesis and increased acyl-glycerol turnover*. Sci Rep, 2017. **7**(1): p. 8983.
126. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method*. Methods, 2001. **25**(4): p. 402-8.

127. Liu, Y., A. Beyer, and R. Aebersold, *On the Dependency of Cellular Protein Levels on mRNA Abundance*. Cell, 2016. **165**(3): p. 535-50.
128. Maier, T., M. Guell, and L. Serrano, *Correlation of mRNA and protein in complex biological samples*. FEBS Lett, 2009. **583**(24): p. 3966-73.
129. Roederer, M., *Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats*. Cytometry, 2001. **45**(3): p. 194-205.
130. Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man*. Diabetologia, 1985. **28**(7): p. 412-9.
131. Salgado, A.L., et al., *Insulin resistance index (HOMA-IR) in the differentiation of patients with non-alcoholic fatty liver disease and healthy individuals*. Arq Gastroenterol, 2010. **47**(2): p. 165-9.
132. Gayoso-Diz, P., et al., *Insulin resistance (HOMA-IR) cut-off values and the metabolic syndrome in a general adult population: effect of gender and age: EPIRCE cross-sectional study*. BMC Endocrine Disorders, 2013. **13**(1): p. 47.
133. Hagstrom-Toft, E., et al., *Evidence for a major role of skeletal muscle lipolysis in the regulation of lipid oxidation during caloric restriction in vivo*. Diabetes, 2001. **50**(7): p. 1604-11.
134. Gogebakan, O., et al., *GIP increases adipose tissue expression and blood levels of MCP-1 in humans and links high energy diets to inflammation: a randomised trial*. Diabetologia, 2015. **58**(8): p. 1759-68.
135. Hammarstedt, A., T.E. Graham, and B.B. Kahn, *Adipose tissue dysregulation and reduced insulin sensitivity in non-obese individuals with enlarged abdominal adipose cells*. Diabetol Metab Syndr, 2012. **4**(1): p. 42.
136. Jernas, M., et al., *Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression*. FASEB J, 2006. **20**(9): p. 1540-2.
137. Hammarstedt, A., et al., *WISP2 regulates preadipocyte commitment and PPARgamma activation by BMP4*. Proc Natl Acad Sci U S A, 2013. **110**(7): p. 2563-8.
138. Hammarstedt, A., et al., *Impaired Adipogenesis and Dysfunctional Adipose Tissue in Human Hypertrophic Obesity*. Physiol Rev, 2018. **98**(4): p. 1911-1941.
139. Berry, R., et al., *Adipose Tissue Residing Progenitors (Adipocyte Lineage Progenitors and Adipose Derived Stem Cells (ADSC))*. Curr Mol Biol Rep, 2015. **1**(3): p. 101-109.
140. Gao, H., et al., *CD36 Is a Marker of Human Adipocyte Progenitors with Pronounced Adipogenic and Triglyceride Accumulation Potential*. Stem Cells, 2017. **35**(7): p. 1799-1814.
141. Rodeheffer, M.S., K. Birsoy, and J.M. Friedman, *Identification of white adipocyte progenitor cells in vivo*. Cell, 2008. **135**(2): p. 240-9.
142. Secco, B., et al., *Amplification of Adipogenic Commitment by VSTM2A*. Cell Rep, 2017. **18**(1): p. 93-106.
143. Baglioni, S., et al., *Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue*. FASEB J, 2009. **23**(10): p. 3494-505.
144. Kim, B., et al., *Gene expression profiles of human subcutaneous and visceral adipose-derived stem cells*. Cell Biochem Funct, 2016. **34**(8): p. 563-571.

145. Tchkonina, T., et al., *Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes*. Am J Physiol Regul Integr Comp Physiol, 2002. **282**(5): p. R1286-96.
146. Toyoda, M., et al., *Characterization and comparison of adipose tissue-derived cells from human subcutaneous and omental adipose tissues*. Cell Biochem Funct, 2009. **27**(7): p. 440-7.
147. Chan, P.C., et al., *Targetted inhibition of CD74 attenuates adipose COX-2-MIF-mediated MI macrophage polarization and retards obesity-related adipose tissue inflammation and insulin resistance*. Clin Sci (Lond), 2018. **132**(14): p. 1581-1596.
148. Nissen, S.E. and K. Wolski, *Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes*. N Engl J Med, 2007. **356**(24): p. 2457-71.
149. Cheng, D., H. Gao, and W. Li, *Long-term risk of rosiglitazone on cardiovascular events: a systematic review and meta-analysis*. Endokrynol Pol, 2018.
150. Stone, J.C., et al., *Was there really any evidence that rosiglitazone increased the risk of myocardial infarction or death from cardiovascular causes?* Pharmacoepidemiol Drug Saf, 2015. **24**(3): p. 223-7.

